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Characterization of Tissue Factor Expression on the Human Endothelial Cell Line ECV304

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The endothelial cell line ECV304 is a spontaneously transformed cell line established from human umbilical vein. The characterization of tissue factor (TF) expression by ECV304 cells has been accomplished in this study. ECV304 cells expressed both TF mRNA and antigen (TF_{ag}) constitutively. In ECV304 cell lysates, the levels of TF_{ag} (1.4 ± 0.3 ng of $TF_{ag}/10^6$ cells) were considerably higher than in THP-1 monocytoid cells (0.07 ± 0.03 ng of $TF_{ag}/10^6$ cells). TF_{ag} was also detected on the ECV304 cell surface by flow cytometric studies. In binding analyses, $3.5 \pm 0.7 \times 10^4$ molecules of TF per cell were estimated, similar to the amounts found in ECV304 cell lysates ($2.9 \pm 0.6 \times 10^4$ molecules/cell), suggesting that all TF_{ag} was translocated to the cell surface. Phorbol myristate acetate (PMA) stimulation of ECV304 cells resulted in an increase of TF mRNA levels, which was abrogated when gene transcription was impaired, suggesting a transcriptional regulation of the TF gene by PMA. In contrast, TF_{ag} was not elevated by PMA-stimulation, indicating the existence of additional posttranscriptional mechanisms. Thus, ECV304 cells constitute a singular endothelial cell model for exploring the regulation of TF expression. Am. J. Hematol. 56:71–78, 1997. © 1997 Wiley-Liss, Inc.

Key words: tissue factor; endothelial cells; tissue factor expression; procoagulant activities; cell lines; tissue factor regulation; phorbol esters

INTRODUCTION

Tissue factor (TF) is a membrane-anchored glycoprotein which catalyzes factor X and factor IX activation by factor VII on cell surfaces (reviewed in Ruf and Edgington [1]. Binding of factor VII to TF induces a 10^6-fold increase in the catalytic efficiency of factor X and IX activation [1–3]. TF is encoded by a 12.4-kbp gene on human chromosome 1 (reviewed in Markman [4]). Inactivation of the TF gene in transgenic mice induces embryonic lethality, related to an abnormal vascular development [5]. TF expression is differently and highly regulated in tissues [1,2,4,6–9]. Some organs, for example, constitutively express TF, such as the cerebral cortex, heart, and renal cortex. TF is also constitutively expressed by some ''envelope'' tissues such as epithelium, synovial, and mucosae [6]. Conversely, under normal conditions, TF is not expressed by cells of the vascular compartment [6]. However, several inflammatory mediators, such tumor necrosis factor α (TNFα), interleukin-1β, and endotoxin (lipopolysaccharide, LPS), as well as mechanical disruption, many induce a localized expression of TF in a functionally active form on monocytes and epithelial cells [1,10–17]. Thus, throughout this pathway, in response to injury TF can trigger blood coagulation in precise and localized areas of the vasculature.

Human endothelial ECV304 cells are spontaneously

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transformed human umbilical vein endothelial cells which have been established and maintained for more than 10 years in culture [18]. Although these cells lack some of the characteristics of HUVEC, for example, they do not express von Willebrand factor [18], but they display other endothelial cell constituents such as typical Weibel-Palade bodies of endothelial cells, they bind to the lectin from *Ulex europeus* I, and they express PHM5 and ICAM-1 [18,19]. ECV304 cells are also endowed with angiotensin-converting enzyme activity and are negative for alkaline and acid phosphatase as well as for some epithelial cell markers such as keratin [18]. ECV304 cells are immortalized and can be grown very easily and without special requirements, expressing some receptors with a constant capacity and affinity from passage to passage [20,21].

Although the phenotypic characterization of ECV304 cell constituents involved in hemostasis is still in progress, the characterization of fibrinolytic components has recently been reported. ECV304 cells produce tissue-type plasminogen activator (tPA), type 1 plasminogen activator inhibitor (PAI-1), and large amounts of single-chain urokinase [21–23]. ECV304 cells also display receptors for plasminogen and tPA [20] as well as for uPA (personal observation). Thus, these cells may be utilized in studies on the cellular interactions of fibrinolytic components. In contrast, no information is currently available on the characterization of TF expression by these cells.

The expression of TF by ECV304 cells has been analyzed in this paper. We have found that, in contrast to HUVEC and to human endothelial cells, nonstimulated ECV304 cells express large amonts of functional TF on their cell surface.

MATERIALS AND METHODS

Proteins and Antibodies

Relipidated recombinant human TF was kindly provided by Dr. T. S. Edgington (Scripps Research Institute, La Jolla, CA). The monoclonal antibody TF4503, factor VI, factor X, and Spectrozyme FXa were from American Diagnostica, Inc. (Greenwich, CT). The monoclonal antibody anti-human plasminogen (clone 2E1) was generated in our laboratory [24]. Anti-TF and anti-plasminogen monoclonal antibodies were radiolabeled using a modified chloramine T method [25]. Phycoerythrin (PE)-conjugated goat anti-mouse Ig was from Sera-Lab, Ltd. (Grawley Down, UK).

Cells

The human endothelial cell line ECV304 was a gift from Dr. K. Takahashi (National Defense College, Tokorazawa, Japan). The human pre-B leukemia cell line Nalm6 was provided by Dr. J. Inglés-Esteve (IRO, Barcelona, Spain). U937 and THP-1 cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The human endothelial cell line ECV304 and the lymphoid cell line Nalm6 (pre-B leukemia) were cultured in RPMI-1640 (Bio-Wittaker/MA Byproducts, Walkerville, MD) containing 1 mmol/l Na pyruvate and 10% fetal bovine serum (FBS, Bio-Wittaker). The human monocytoid cell line THP-1 was grown in RPMI-1640 containing 1 mmol/l Na pyruvate, 0.05 mmol/l HEPES, pH 7.35, and 5% FBS. The human monocytoid cell line U937 was cultured in RPMI-1640 with 5% FBS.

Northern Blot Analysis

Total RNA was isolated using a commercial kit, Ultraspec™ RNA (Biotecx, Houston, TX), according to the manufacturer's instructions. Twenty micrograms of total RNA were fractionated by electrophoresis on 1% formaldehyde-agarose gel and transferred to nylon membranes (Boehringer Mannheim, Mannheim, Germany). The membranes were prehybridized for 4 hr at 42°C, in a solution containing 50% formamide, 5 × SSC, 50 mmol/l sodium phosphate, pH 7.0, 5 × Denhardt's solution, 1% glycine, 0.1% SDS, and 350 μg/ml salmon sperm DNA. Hybridization was performed in a rotation over at 42°C with a 775-bp *Eco*RI fragment from plasmid pETF 1773 [26], containing the human TF cDNA [4] that had been radiolabeled with (α^{32}P)dCTP using a Multiprime Labeling Kit® (Amersham, Buckinghamshire, UK). After 16 hr, the membranes were subjected to two 15-min washes at room temperature in 2 × SSC, 0.1% SDS, and two extra 15-min washes at 65°C in 0.1 × SSC, 0.1% SDS. Membranes were exposed between 12–48 hr for autoradiography.

TF Antigen in Cell Lysates

To measure the amount of TF present within ECV304 cells, cells were grown to confluence in 12-well tissue culture plates, detached by a 15-min treatment with 0.5 mmol/l EDTA, washed, and counted. The cells were resuspended and incubated for 10 min at 37°C in 0.1% Triton X-100 solution. Then, cell lysates were diluted in 20 mmol/l Tris HCl buffer, pH 7.35, 130 mmol/l NaCl, and 0.3% Tween-80. The TF protein content in cell lysates was measured using a commerically available enzyme-linked immunoabsorbent assay (ELISA) for TF (IMMUBIND® TF, American Diagnostica, Inc.).

Flow Cytometric Analyses

For flow cytometric analyses, ECV304 cells were detached by a 15-min treatment with 0.5 mmol/l EDTA and then washed (×2) with PBS (0.01 mol/l Na phosphate, 0.14 mol/l NaCl, pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (PBA). Cell suspensions were incubated for 30 min at 4°C with PBA containing 10% inactivated rabbit serum, washed again,

and then incubated for 1 hr at 4°C with 15 nmol/l of either anti-TF or anti-plasminogen (negative control). After two washes with PBA, cells were stained for 30 min at 4°C with PE-labeled goat anti-mouse IgG at a 1/100 dilution. Bound IgGs were analyzed in a flow cytometry analyzer (Coulter's EPICS Profile II, Hialeah, FL).

Ligand-Binding Analyses

For binding analyses of the human endothelial ECV304 cell line, cells were plated in 12-well culture plates. The cells were washed three times in Hanks' balanced salt solution (Imperial Laboratories, Hants, UK) containing 1.2 mmol/l $CaCl_2$, 1.6 mmol/l $MgSO_4$, and 0.05 mol/l HEPES, pH 7.35 (HBSS-HEPES), and resuspended in HBSS-HEPES containing 0.1% BSA (HBSS-HEPES-BSA), followed by addition of radiolabeled ligand (10 nmol/l), buffer, and competitors to a final volume of 400 μl. After incubation for 1 hr at 4°C, wells were washed three times with HBSS-HEPES-BSA, and the cells were solubilized with 250 μl 0.1% SDS in H_2O]27] and counted for radioactivity. Molecules of ligand bound per cell were calculated based upon the specific activities of radiolabeled ligands. Specific binding was defined as that component of bound radioactivity that could be inhibited by a 65-fold molar excess of unlabeled ligand. Binding of BSA-coated wells was only minimally inhibited by unlabeled ligand (10–15%). Total nonspecific binding for ECV304 cell-containing wells did not exceed 25% at any given antibody concentration.

Tissue-Factor Activity

Tissue-factor activity in lysed cells was determined by a continuous chromogenic assay, as described previously [28,29]. Briefly, 60 μl of lysated cells in Tris-saline, containing 1 mg/ml BSA, were added to 96-well culture plates (Falcon, Seville, Spain). The following reagents were sequentially added to each well: 20 μl of 1 nM factor VIIa, 20μl of 30 nM factor X, 10 μl of 5 mM Spectrozyme FXa, and 50 μl of 25 mM $CaCl_2$. Absorbance (at 405 nm) of each well was determined at 1–3-min intervals over a period of 80 min using an ELISA reader (Organon Teknica, Barcelona, Spain). Relipidated recombinant human TF (4–500 pg/ml) was used as the standard. A calibration curve was prepared by plotting TF-concentration vs. absorbance on a logarithmic plot. In this assay 50 pg/ml of recombinant human TF was arbitrarily designated as corresponding to 100 units of procoagulant activity. Factor X activation, occurring independently of tissue factor, were measured by omitting factor VII from the reaction mixtures and substracted.

Other Reagents

BSA, Triton-X 100, PMA (12-O-tetradecanolyphorbol-1,3-acetate), actinomycin D, and cycloheximide were from Sigma (St. Louis, MO). Trypsin-EDTA solution was from Gibco-BRL (Paisley, Scotland, UK).

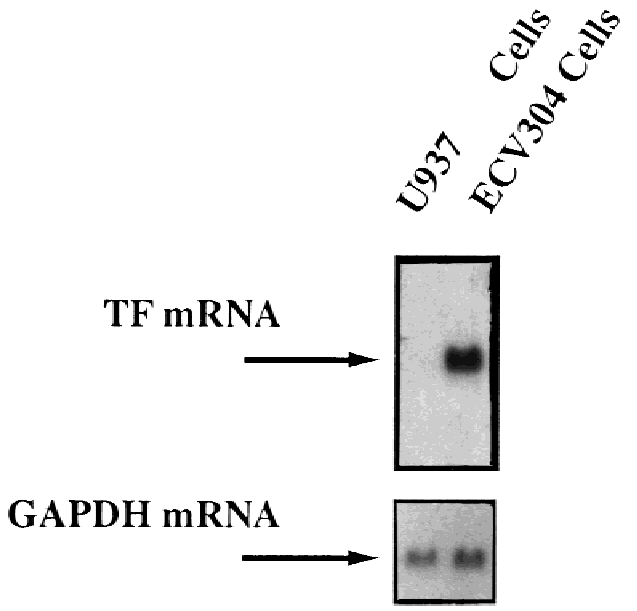

Fig. 1. TF mRNA expression in nonstimulated ECV304 cells. ECV304 and U937 cells were grown in endotoxin-free media. Twenty micrograms of total RNA from both cell lines were analyzed for TF mRNA expression by Northern blotting. GAPDH mRNA was analyzed as an internal control for loading.

RESULTS

TF Expression in ECV304 Cells

To initially assess whether ECV304 cells express TF mRNA, samples of total RNA were analyzed for TF mRNA expression by Northern blotting. Figure 1 shows the presence of a specific TF mRNA in nonstimulated ECV304 cells, whereas U937 monocytoid cells, used as a negative control, showed no detectable TF mRNA. TF is not normally expressed by nonstimulated cells within the vasculature; however, some agents such as endotoxin (LPS) can induce TF expression by monocytes and endothelial cells. Therefore, we wanted to known whether the basal expression of TF in nonstimulated ECV304 cells was due to the presence of endotoxin in the culture media. The quantitation of endotoxin in culture media was assessed as previously described [30], using a commercially available kit (Coatest® Endotoxin, Kabi, Stockholm, Sweden). The amount of LPS detected in supernatants of ECV304 cells was lower than background levels (<0.02 EU/ml). In addition, the U937 cells were grown in parallel to ECV304 cells in the same meida preparations and were used in the Northern blot experiments. This initial experiment suggested that nonstimulated ECV304 cells expressed RF mRNA.

To determine whether this observation could also be

TABLE I. Comparison of TF_{ag} Levels in Different Cell Lines*

Cell line	Cell type	TF_{ag} (pg/10^6 cells)	TF_{ag} (molecules/cell) × 10^4	Binding of ^{125}I-anti-TF (molecules/cell) × 10^4
ECV304	Endothelial	1,410 ± 325	2.97 ± 0.66	3.5 ± 0.7
THP-1	Monocytoid	74 ± 26	0.14 ± 0.05	0.6 ± 0.2
Nalm6	Lymphoid	19 ± 11	0.03 ± 0.01	0.1 ± 0.1

*TF_{ag} in cell lysates was measured by ELISA, and molecules of cell surface-associated TF_{ag} were determined as described in Materials and Methods. The amounts of TF_{ag} in ECV304 cells were compared to those found in nonstimulated THP-1 (monocytoid) and Nalm6 (lymphoid) cell lines. Mean ± SD of 3–6 separate experiments.

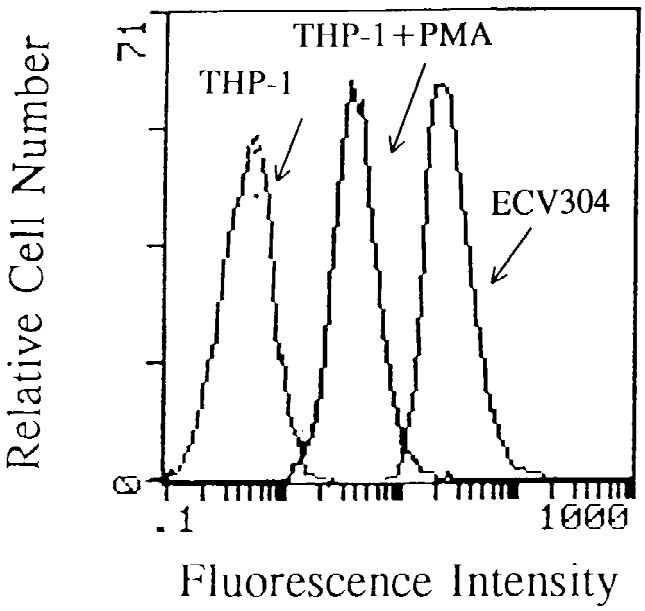

Fig. 2. Flow cytometry analyses of cell surface-associated TF_{ag}. Nonstimulated ECV304 cells were analyzed by flow cytrometry using a monoclonal antibody against human TF. The histogram obtained with these cells was compared to those of nonstimulated and PMA-stimulated (10 nmol/l for 5 hr) THP-1 monocytoid cells. Negative controls for fluorescence intensity (using an anti-plasminogen monoclonal antibody or without first antibody) for each cell type overlapped with the histogram for nonstimulated THP-1 cells.

confirmed in protein assays, the presence of TF protein in ECV304 cells was explored at two different levels. TF_{ag} (TF antigen) was measured in cell lysates of ECV304 cells and the results compared to those found in lysates of the monocytoid cell line THP-1 and the lymphoid cell line Nalm6. Results are summarized in Table I. The amount of TF_{ag} detected in ECV304 cells was between 4–14-fold higher than that detected on nonstimulated THP-1 cells, whereas TF_{ag} was undetectable in the lymphoid cell line Nalm6 (negative control). To determine whether TF_{ag} in ECV304 cells was cell surface-associated, those cells were detached by EDTA treatment and analyzed by flow cytometric analysis. ECV304 cells were positive for TF (Fig. 2), whereas nonstimulated THP-1 were negative. ECV304 cells expressed higher levels of TF than THP-1 cells, which were stimulated for 5 hr with 10 nmol/l PMA, indicating that a single population of nonstimulated ECV304 cells expressed high amounts of TF on its cell surface. ECV304 cells were detached by a short-term EDTA treatment. To determine whether EDTA could modify TF expression in THP-1 cells, these monocytoid cells were treated with EDTA in conditions identical to those for ECV304 cells and analyzed for TF expression by FACS analysis and for TF procoagulant activity. No differences between EDTA-treated and untreated cells were observed by FACS analysis (data not shown), and only a slight increase (≈6%) in TF procoagulant activities was observed after EDTA treatment (155 ± 20 and 164 ± 30 U/10^6 cells of TF procoagulant activity were found in untreated and EDTA-treated THP-1 cells, respectively). No differences were observed in the expression of TF on cell surfaces when ECV304 or THP-1 cells were grown in media containing either 5% or 10% FBS.

Quantitation of the number of TF cell surface-associated molecules per cell was assessed by binding analyses. In these studies, a ^{125}I-radiolabeled monoclonal antibody for TF was used as ligand and added to adherent ECV304 cells. In Western blot analyses, this antibody recognized a single band of 45–50 kDa when, following electrophoresis, purified TF or ECV304 cell lysates were transferred to nitrocellulose sheets. This 45–50-kDa component was not detected in cell lysates from Nalm6 cells. The reactivity of this antibody was abrogated by preincubation of the antibody with purified TF (data not shown). The apparent Kd of this antibody for TF was 60 ± 20 pmol/l. Thus, in subsequent binding experiments, the radiolabled ligand was added at a 10 nmol/l final concentration, and incubated at 4°C for 1 hr with the cells (2–4 × 10^5 cells/ml). The specificity of binding of the radiolabeled anti-TF to ECV304 cells was determined by adding as competitor an excess of unlabeled antibody at three orders of magnitude (650 nmol/l) over the apparent Kd to mixtures of ^{125}I-anti-TF and cells. After incubation, cells were washed, lysed, and counted for radioactivity. Molecules of radiolabeled ligand bound per cell were calculated based upon the specific activity of the radiolabeled ligand. ECV304 cells bound 3.5 ± 0.7 × 10^4 molecules of radiolabeled anti-TF per cell (mean of six experiments) and <800 molecules per cell of ^{125}I-radiolabeled monoclonal antibody anti-plasminogen 2E1, produced in our laboratory [24] and used as negative control. These data indicated that a large number of TF molecules were translocated to the cell surface of nonstimulated ECV304 cells and were recognized by monoclonal antibody anti-TF 4503.

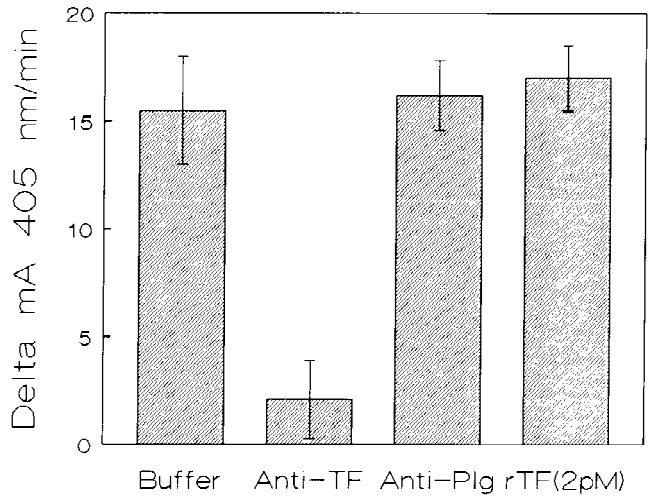

Fig. 3. Functionality of TF in ECV304 cells. TF procoagulant activity (TF-PCA) was measured in ECV304 cells by a continuous chromogenic assay, as described in the text. In comparison, TF-PCA was also determined in THP-1 and Nalm6 cells.

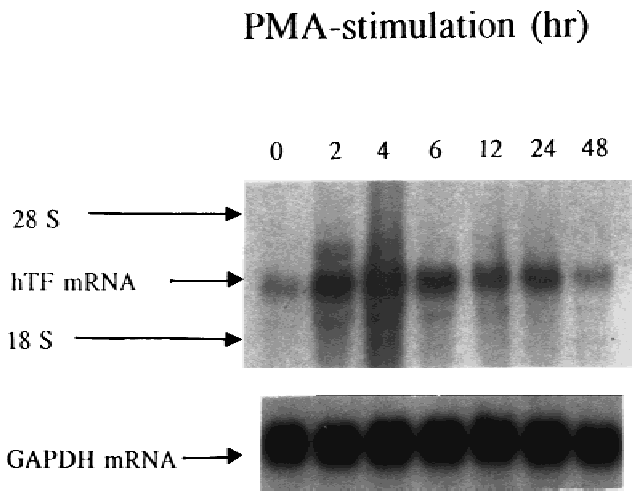

Fig. 4. Time-course analysis of TF mRNA expression in PMA-stimulated ECV304 cells. Twenty micrograms of total RNA from nonstimulated and PMA-stimulated ECV304 cells were analyzed for TF mRNA expression at the indicated times by Northern blotting. GAPDH mRNA was analyzed as an internal control for loading.

With the evidence that TF was expressed on the cell surface of ECV304 cells, we analyzed whether TF was in an active form. Thus, TF procoagulant activity (TF-PCA) was analyzed in continuous chromogenic assay [28,29]. As shown in Figure 3, ECV304 cells exhibited a high TF-PCA (3,255 ± 380 TF-PCA U/10^6 cells) in comparison to THP-1 or Nalm6 cells (155 ± 20 and <10 TF-PCA U/10^6 cells, respectively). The expression of TF-PCA in ECV304 cells ranged from 3,635–2,875 TF-PCA U/10^6 cells, indicating that this endothelial cell line expressed an 18–23-fold higher TF-PCA than did monocytoid THP-1 cells.

Modulation of TF Expression in ECV304 Cells

The primary regulation of TF expression in monocytes and endothelial cells appears to be transcriptional [1,16,31–35], although posttranscriptional mechanisms are also involved [33,36,37]. PMA has been shown to induce TF mRNA and protein in a wide variety of cells, including THP-1 and HeLa cells as well as HUVECs [1,36,38–42]. Similarly, we wanted to investigate whether PMA was able to regulate TF expression in ECV304 cells. ECV304 cells were incubated with 10 nmol/l PMA for various periods of time ranging from 0–48 hr. The results, shown in Figure 4, indicate that PMA caused an increase in TF mRNA already detectable at 2 hr and maintained during 48 hr. The peak response was measured at 4 hr.

To further investigate the general mechanism responsible for this increase in TF mRNA upon PMA-stimulation of ECV304 cells, cells were incubated with PMA (10 nmol/l) in the presence or absence of mRNA and protein synthesis inhibitors, such as actinomycin D and cycloheximide, respectively. As shown in Figure 5, treatment of ECV304 cells with PMA alone for 5 hr caused an increase in TF mRNA. This transcript was also incuded by cycloheximide, as previously described by others [43], whereas combinations of both drugs caused an additive effect. In contrast, incubation of ECV304 cells with actinomycin D did not significantly change the basal TF level, whereas stimulation of TF by PMA was completely abolished by this agent.

Interestingly, PMA-stimulation of ECV304 cells did not increase the amount of TF_{ag}, which was maintained between 1.68–1.80 ng/10^6 cells, in a time period ranging from 0–48 hr PMA-stimulation. Therefore, the increase of TF mRNA did not result in an increase in the amount of synthesized TF_{ag}.

DISCUSSION

Some phenotypic characteristics of ECV304 cells have been described in previous studies [18,19,23,44–48], including the expression of fibrinolytic components [20–23]. We investigated whether ECV304 cells can express TF. To test this possibility we used three general approaches: 1) detection of TF mRNA by Northern blotting; 2) quantitation of TF protein in cell lysates; and 3) presence of TF on the cell surfaces of ECV304 cells.

Using the first approach, we found TF mRNA was detectable even in nonstimulated cells, suggesting that in HUVECs and other human endothelial cells, nonstimulated cells, suggesting that in HUVECs and other human endothelial cells, nonstimulated ECV304 cells express TF mRNA. With the evidence that ECV304 cells tran-

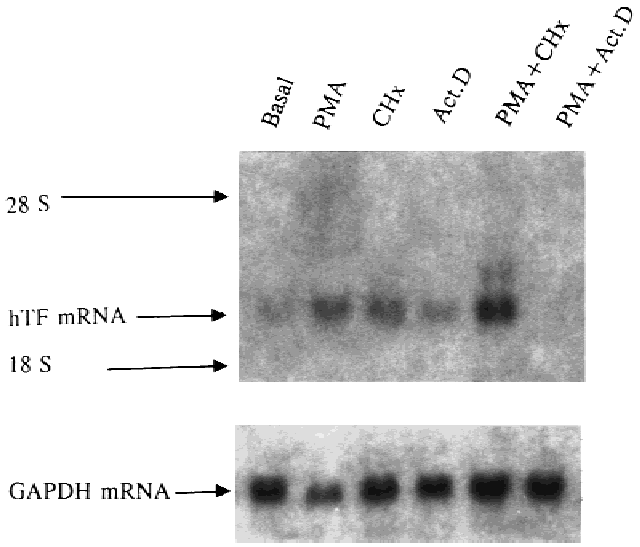

Fig. 5. Modulation of TF mRNA expression in ECV304 cells. Cells were treated with different drugs, alone or in combination, for 5 hr, and 20 µg of total RNA were analyzed by Northern blotting for TF mRNA expression. GAPDH mRNA was used as loading control. Drugs were used at the following concentrations: PMA, 10 nmol/l; cycloheximide (CHx), 50 µg/ml; actinomycin D (Act.D), 5 µg/ml. CHx and Act.D were added to the cell culture 30 min prior to PMA-treatment.

scribe the TF gene, we measured the amount of TF_{ag} in cell lysates of ECV304 cells and compared it to the amount found in lysates from other cell lines. ECV304 cells had a high content of TF_{ag}, ≈1–2 ng/10^6 cells, whereas the levels of TF_{ag} in nonstimulated or 6-hr PMA-stimulated THP-1 cells were 0.074 ± 0.026 and 0.46 ± 0.098 ng/10^6 cells, respectively.

With the third approach, we explored whether TF is translocated to the cell membrane of ECV304 cells. Cell surface-associated TF was detected by flow cytometric analyses, and the number of molecules of TF expressed by cells was estimated from binding assays, using radiolabeled anti-TF as ligand. WIth this method we calculated that ECV304 cells express on their cell surface 3.5 × 10^4 molecules of TF per cell, a number ≈2-fold higher than that found on stimulated peripheral blood monocytes [31]. ECV304 cells do not need to be stimualted to express cell surface-associated TF. Based on the amount of TF detected in ECV304 cell lysates and in binding assays, the number of molecules of TF expressed on the cell surface (≈3.5 × 10^4 molecules per cell) is similar to that detected by ELISA (≈2.97 × 10^4 molecues of TF_{ag} per cells), suggesting that all the TF in ECV304 cells is cell surface-associated, in agreement with data published using other cell types [2]. On ECV304 cells, cell surface-associated TF was endowed with the capacity to bind factor VII. Based upon a tissue-factor proaculant activity assay we found very high amoutns of TF-PCA in resting ECV304 cells. The TF-PCA in ECV304 cells was ≈20-fold higher than that found in unstimulated THP-1 cells, suggesting that TF in ECV304 cells in functionally active.

The TF gene, an immediate-early gene (reviewed in Mackman [4]), is induced rapidly in various cell types in response to different stimuli, including phorbol myristate acetate [32–34,42]. In endothelial cells, although post-transcriptional mechanisms have been suggested [33], the TF gene can be transcriptionally regulated by LPS, TNFα, interleukin-1β, and PMA [4,32–34,49]. In monocytoid and endothelial cells, interactions between the TF enhancer and the Fos-Jun and c-Rel-p65 heterodimers mediate the induction of TF gene transcription [4,35,49]. We explored the effect of PMA on TF mRNA levels in ECV304 cells, which distinctly to HUVECs, express large amounts of TF_{ag} on their cell surface. Although TF transcripts were detected in nonstimulated cells, PMA-stimulation resulted in a rapid increase in steady-state levels of TF mRNA, detectable at 2 hr, maximal at 4 hr, and decreasing after 48 hr. Treatment of ECV304 cells with the protein synthesis inhibitor cycloheximide resulted not only in an upregulation of TF mRNA levels in nonstimulated cells, but also had an additive effect on PMA-mediated TF gene expression, as demonstrated in other cell types [42].

CONCLUSIONS

This study constitutes the first attempt to characterize TF mRNA and protein expression in endothelial ECV304 cells. Our results stress the differences between HUVECs and the transformed ECV304 cells for the expression of this molecule. While parental human umbilical vein endothelial cells do not express TF on the cell surface in resting conditions, the derivative ECV304 cell lines express TF constitutively. However, TF mRNA levels can be elevated upon PMA treatment of ECV304 cells, similar to the induction occurring in HUVECs. This TF mRNA increase is not concomitantly followed by higher TF_{ag} expression, suggesting the existence of posttranscriptional mechanisms regulating overall TF synthesis. The combined action of transcriptional and posttranscriptional processes, which may account for the expression of TF render ECV304 cells as a particular cellular model to study TF gene expression.

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